Low Dose Radiation Injury and Radioprotection in the Vertebrate Embryo

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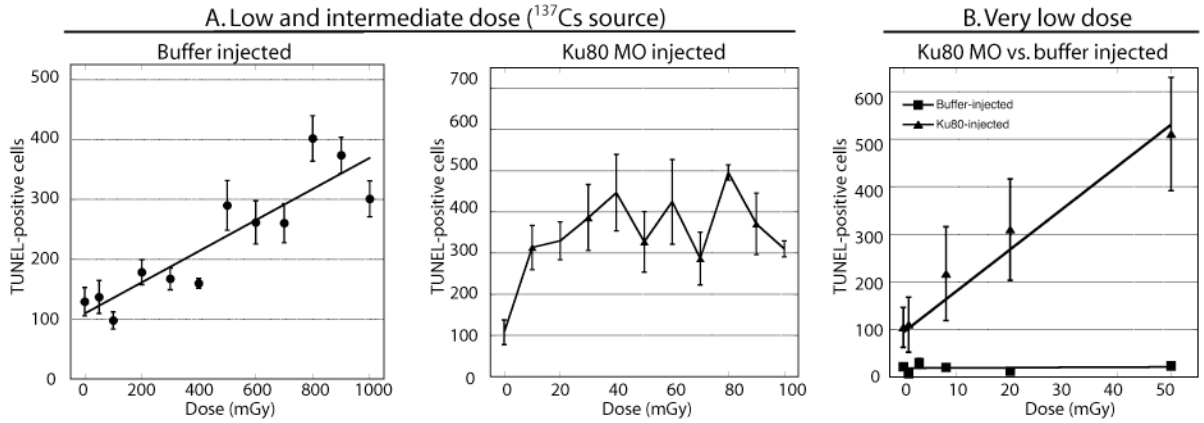
The effects of radiation on vertebrate embryos are of special interest in low-dose radiobiology. Because dividing cells are inherently radiosensitive, and the proportion of dividing cells is higher in an embryo than an adult, the effects of radiation may be correspondingly greater. In addition, morphogenesis is very active in embryos, whereas it occurs to a limited extent, if at all, in adults. Morphogenesis requires coordinated cell division and migration, and disruption of these processes by radiation leads to gross morphological defects. Thus, not only are embryos potentially more sensitive to radiation at the cellular level, but cellular damage may have a greater chance of being translated into injury at the organismal level. We describe here work using the embryos of the zebrafish, *Danio rerio*, as our model system. Zebrafish embryos are small (<1 mm), optically transparent, and develop outside the mother. Zebrafish have many of the organ systems that respond acutely to radiation injury in mammals (hematopoietic system, gut, kidney, and central nervous system). They also have homologues of most mammalian DNA damage detection and repair genes. We describe three aspects of the work:

1. Cloning and characterization of zebrafish DNA repair genes. We isolated cDNA clones encoding the two subunits of Ku protein, which functions in the first step of the nonhomologous end-joining pathway of DNA double-strand break repair. The domain structure of zebrafish Ku70 and Ku80 subunits is similar to that of other vertebrate Ku80 proteins. Maternal Ku80 mRNA is present and uniformly distributed prior to the onset of transcription of the zygotic genome. Ku80 mRNA levels increase substantially after the onset of zygotic transcription, and mRNA accumulation becomes spatially restricted. At 24 hours post fertilization (hpf), mRNA is concentrated in the developing retina, anterior central nervous system (CNS) and otic vesicle, pronephric ducts, and mesenchymal cells located ventrally in the tail. Domains of Ku80 expression in the CNS correspond closely with proliferative regions. Subsequent studies with Ku70 led to similar findings: maternal Ku70 mRNA is uniformly distributed, whereas zygotically transcribed mRNA shows tissue-specific accumulation at later developmental times. To investigate embryonic function of Ku70 and Ku80, we injected 5 ng of Ku80 antisense morpholino oligonucleotide (Ku80 MO) at the 1-cell stage to suppress translation of endogenous Ku80 mRNA. Embryos that received a combination of Ku80 MO and radiation showed a dramatic increase in the number of TUNEL-positive cells compared with embryos that received radiation alone. Cell death was p53-dependent. Microinjection with Ku70 MO resulted in radiosensitization similar to that with Ku80.

2. Quantification of the effects of low dose radiation in the vertebrate embryo. Many radiobiological questions can only be addressed quantitative dose-response data, which motivated us to adapt the TUNEL staining approach for quantitative studies. We exposed embryos to controlled low doses of radiation at 6 hpf, when the embryonic cell population is morphologically uniform and cells are not yet committed to a specific fate. At 24 hpf, we performed fluorescent TUNEL staining to detect apoptotic cells, collected confocal image stacks, and performed three-dimensional reconstruction to determine the number and distribution of TUNEL-positive cells. The distribution of TUNEL-positive cells was uniform, consistent with a cell-autonomous mechanism of cell death. The dose response was approximately linear, as predicted for this dose range by a linear quadratic model. There was no evidence for low dose radiation hypersensitivity or induced radioresistance, which would have led to significant deviation from linearity. Microinjection with Ku80 antisense oligonucleotide leads to a dose reduction factor of 35-fold, confirming the importance of nonhomologous end joining in radioprotection. Results illustrate the potential of the vertebrate embryo for automated, quantitative studies of radiation-induced cell death in the context of an intact organism.



Quantification of radiation dose response in the embryo. A. Dose response in low and intermediate dose range. Irradiation of buffer and Ku80 morpholino-microinjected embryos was performed using ^{137}Cs (Gammacell Exactor, MDS Nordion, Ontario, Canada) at 6 hpf. TUNEL staining was performed at 24 hpf. All embryos are from the same clutch and were treated in parallel. Experimental groups generally contained at least eight embryos. Bars denote standard error of the mean. B. Dose response curves in the very low dose range. Embryos were treated as in A except that irradiation was performed using a 6 MeV Varian linear accelerator beam, which was attenuated and calibrated to deliver an accurate dose in the 1 to 50 mGy range. shown.

3. *Effects of oxidative stress and low dose ionizing radiation: same or different?*

It has been proposed that effects of reactive oxygen species (ROS) generated by low dose radiation exposure might, in effect, become lost against the background of endogenous ROS produced by normal metabolic processes in aerobically-living organisms. To investigate whether low dose radiation injury was the same or different from other forms of oxidative stress, we induced ROS in embryos by treatment with menadione ((2-Methyl-1,4-naphthoquinone), a form of Vitamin K. Menadione has been widely used in studies of oxidative stress. It undergoes an intracellular enzymatic one-electron reduction, using endogenous flavoproteins as the donor, to form a semiquinone radical. This in turn rapidly reduces O_2 to superoxide, which can form more toxic ROS, including hydroxyl radical. The reaction regenerates the quinone, which can then enter into a new cycle of ROS production.

To obtain baseline information about the effects of menadione alone, we exposed 6 hpf embryos exposed to various concentrations of menadione for 3 h. Menadione was removed and embryos were allowed to develop. Treatment with 3 μM menadione induced persistent oxidative stress, as detected in living embryos using Image-iT live (Molecular Probes) staining. This was accompanied by pronounced, but transient, developmental delay. Treatment with 10 μM menadione produced a more severe developmental delay and in some experiments was lethal.

Embryos were then treated with combinations of radiation and menadione at various doses. Phenotypes were clearly distinct: for example, treatment with 150 cGy of ionizing radiation produced some apoptosis but little morphologic abnormality, whereas treatment with 3-10 μM menadione produced developmental delay but little apoptosis. The effect of combined treatment with intermediate doses of radiation and menadione appeared to be more than additive. Significantly, although attenuation of Ku80 function by oligonucleotide microinjection sensitized the embryos to low dose radiation, it did not further increase the effects of 10 μM menadione. Together, results indicate that exposure to low doses of ionizing radiation produces effects that are qualitatively different from effects of exposure to reactive oxygen species from endogenous sources.